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DEVELOPMENT AND KINETIC VALIDATION OF BIO-CATALATIC PATHWAY FOR THE QUANTIFICATION OF CATALASE ACTIVITY USING 3-METHYL-2 BENZOTHIAZOLINEHYDRAZONE HYDROCHLORIDE AND PYROCATECHOL AS A CHROMOGENIC CO-SUBSTRATES

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ABSTRACT

A kinetic model describing the assay of catalase (CAT) activity on hydrogen peroxide using pyrocatechol (PC) and 3-methyl-2 benzothiazolinehydrazone hydrochloride (MBTH) is presented. This model is based on the oxidation of PC by H_2O_2 in presence of catalase to form quinone, which couples with oxidized MBTH resulting in intense red chromogenic product having maximum absorbance at 500 nm. Hence the activity of catalase was directly measured by the formation of the coupled product. The catalase assay was drawn-out by the kinetic response of the MBTH-PC system. The quantification of catalase was linear over 0.14–3.46 EU with a correlation coefficient of 0.996. This assay was adopted for the quantification of H_2O_2 between 0.60 and 9.62 mM. The catalytic efficiency, catalytic power and catalytic constant (k_{cat}) of the catalase were 1.16×10^6 M^{-1} min^{-1}, 3.25×10^{-5} min^{-1} and 0.1161 min^{-1}respectively. The method was tested with some microbes and also compared with L. Goth system.

KEYWORDS: Pyrocatechol, 3-Methyl-2 Benzothiazolinehydrazone Hydrochloride, Catalase, Microorganisms

INTRODUCTION

Catalase (H_2O_2: H_2O_2 Oxidoreductase, EC1.11.1.6) is the enzyme commonly present in cells of plants, animals and aerobic bacteria. It is a tetramer of polypeptide chain composed of more than 500 amino acids. Positioned within this tetramer are four porphyrin heme groups, which are very similar to the familiar hemoglobin, cytochromes, chlorophylls and nitrogen fixing enzymes. Catalase are closely related to peroxidases, both structurally and functionally having two functions; it reacts 'peroxidatically' at low concentration of peroxide and 'catalatically' at high concentration of peroxide (I. J. George Wang, et al, 1986). It promotes the conversion of H_2O_2; a powerful and potentially harmful oxidizing agent, to water and molecular oxygen. It protects cells against potential oxidative damage from free radical formation and it has one of the highest turnover rates compared to all enzymes [Van Lenteand, F, et al, 1990, Goth, L. et al, 2003].

Determination of catalase level in biological matrix is based on its activity measurement. In quantitative work, this has referred to the activity of the purified enzyme. Several methods are available for measuring catalase activity and most of them are modifications of the one reported by Chance and Herbert (Ukeda, H, et al, 2004). This method is based on the amount of H_2O_2 decomposed by catalase, which is determined by measuring the decrease in the absorbance of H_2O_2 at 240 nm, which is prone to interferences by other absorbers (Slaughter, M.R. et al, 2000). In this method the absorbance

is measured at very low wavelength region, hence correct absorbance value is not very often obtained due to interferences by endogenous and exogenous UV-absorbing component present in or added to the biological sample. Regrettably, the UV photometric method is also not straightforwardly applicable to complex samples displaying strong intrinsic absorption at 240 nm (Wu. Meng, et al, 2003). The catalase in blood and tissue is quantified by several methods, which include the conventional titrimetric method of analysis (Aebi, H. 1984), micro titer plate assay method (Humphreys, D.T et al, 1999), amperometric method using Clark oxygen electrode (Yamazaki, S.I., et al, 2004), two-mixer quenched - flow technique of Ogura (Ogura, Y. 1955), HPLC method (Bohmer, A. et al, 2011), chemiluminescence (Mueller, S. et al, 1997), cyclic voltammetry (Huang, K. J. et al, 2011), polarographic (Rigo, A. et al, 1977), electrochemical impedance spectroscopy (Shamsipur, M. et al, 2012) and manometric method (Siqueira, A.J.S. et al, 1999). The titrimetric and micro titer plate assays lack accuracy and precession whereas the amperometric method is not economical and tedious. In quenched-flow technique the reaction time is in the range of 0.1-0.4 sec with a very high enzyme concentration (0-12 μM) and in manometric method, catalase activity is determined with a very low enzyme concentration.

The assay of catalase activity at very low catalase concentrations as per Jones and Suggett analysis (Jones, P. et al, 1968) presents severe difficulties, and the work by Irwin W. Sizer (Sizer, I. W., 1944) suggested substantial thermal deactivation of catalase. A systematic kinetic study of catalytic action at high substrate concentration requires both maximal precision in the estimation of extent of reaction and isothermal conditions. (Peter, J.A et al, 1968) Another method developed (Lewis, E.M. et al, 2009) involved the catalytic decomposition of H_2O_2 by using a computer-interfaced pressure sensor to follow the evolution of O_2 (g) inside a closed reaction vessel. This required the electronic pressure-sensor, which is not economical and not easy to handle. HPLC, chemiluminescence, cyclic voltammetry, polarographic, electrochemical impedance spectroscopy and these experiments are either very expensive or less versatile, and moreover the methods are often complicated and expensive to implement. The selectivity of the luminescence is poor. One of the drawbacks of electrochemical sensors is the interference by oxidation or reduction of other compounds at the working potential and also, electro-analytical technique needs several steps to immobilize the enzyme on a solid support, which may reduce the enzyme activity (15 %) resulting in the waste of expensive biocatalyst, and it is also a time consuming process (Mueller, S. et al, 1997).

In the present investigation, an attempt was made to develop a simple, rapid, low cost and sensitive spectrophotometric method for the assay and kinetic study of catalase present in some microbes, by using nontoxic materials like MBTH and PC for the assay of catalase. Therefore this work was aimed at the assay of catalase and H_2O_2 at very narrow range of concentrations.

MATERIALS AND METHODS

Chemical Reagents and Their Preparation

All the chemicals used in the assay were of analytical grade. MBTH and PC were purchased from Sigma-Aldrich and Merck, Germany respectively. MBTH (4.5 mM) and PC (18.0 mM) solutions were prepared by dissolving a requisite quantity in distilled water. Catalase (bovine liver, EC 1.11.1.7, 4326 units/mg) was purchased from Sigma-Aldrich, Germany and its solution was prepared by dissolving 10 mg in 5 mL of distilled water. It was used as a standard stock solution and stored at 4°C. Further dilution was made with double distilled water when required. H_2O_2 (30%) was purchased from E Merck, Mumbai, India. The 8% v/v H_2O_2 stock solution was prepared daily and its concentration was standardized by titration with secondary standard potassium permanganate (Vogel, A.I. 2006). Tris buffer (0.5 M) of

pH 9.8 was prepared by dissolving 1.514 g of tris (hydroxyl methyl) methyl amine [2-amino, 2-(hydroxyl methyl) propane-1-3-diol] in 25 mL using distilled water. Double-distilled water was used throughout the experiment. All solutions were preserved under temperature range of 0-10°C.

Instrumentation

A Jasco model UVIDEC-610 ultraviolet–visible (UV–Vis) spectrophotometer with 1.0-cm matched cells was used for all absorbance measurements. A water bath shaker (NSW 133, New Delhi, India) was used to maintain constant temperature for color development. All pH measurements and adjustments were done by a digital pH meter (model EQ-614, Equip-tronics, Mumbai, India).

Procedure for Inoculum Preparation

At least three to five well-isolated colonies having same morphological types were selected from agar plate culture plates of *Staphylococcus aureus, Bacillus subtilis,* and *Pseudomonas aeruginosa.* The top of each colony was touched with a sterilized loop, and the growth was transferred aseptically into a tube containing 4 to 5 mL of sterile Mueller-Hinton broth medium. The broth culture was incubated at 35°C until it achieved or exceeded the turbidity of 0.5 McFarland standards. McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. The turbidity of the actively growing broth culture was adjusted with sterile Mueller-Hinton broth to obtain turbidity optically comparable to that of 0.5 McFarland standards aseptically. This resulted suspension containing 1 to 2×10^8 bacteria / mL

THEORY

Kinetic Model for the Evaluation of Michaelis-Menten Constant for MBTH and PC

To assess the kinetic parameters, the following experimental conditions were maintained in the reaction: tris buffer of pH 9.8 (50.0 mM), temperature 30°C and reaction time 5 min. The enzyme concentration was maintained at 1.15 EU per 3 mL of reaction mixture. In the presented method, separate experiment for each H_2O_2 concentration was performed with varying concentration of MBTH and PC. Michaelis - Menten constants at concentrations from 0.0570 to 0.1426 mM for MBTH and 0.1513 to 0.6054 mM for PC were determined. The H_2O_2 concentrations of 3.85, 5.77, 7.70 and 9.62 mM with MBTH and 2.40, 4.81, 7.21 and 9.62 mM with PC in the final volume of 3 ml were used for each kinetic study. The pH and temperature were kept constant. The kinetic mechanism followed by catalase can be confirmed by the plot of 1/ $[H_2O_2]$ *versus* V_{max}/V_0 concentrations at different H_2O_2 concentrations. Assuming the initial rate as (V_0), a general equation for the mechanism in the forward direction is given as a function of all substrate concentrations. By rearrangement of Henri-Michaelis-Menten equation into a linear form, we get

$$\frac{1}{V_0} = \frac{1}{V_{\max}} + \frac{K_m^{H_2O_2}}{H_0 V_{\max}} + \frac{K_m^{MBTH}}{M_0 V_{\max}} + \frac{K_m^{PC}}{P_0 V_{\max}}. \quad (1)$$

Rearranging the above equation we get,

$$\frac{V_{\max}}{V_0} = 1 + \frac{K_m^{H_2O_2}}{H_0} + \frac{K_m^{MBTH}}{M_0} + \frac{K_m^{PC}}{P_0}. \quad (2)$$

Evaluation of the Michaelis- Menten constant of the MBTH and PC were carried out by the plot of V_{max}/V_0 and $1/H_0$ with an intercept,

$$Intercept = 1 + \frac{K_m^{MBTH}}{M_0} + \frac{K_m^{PC}}{P_0}. \quad (3)$$

The plot of corresponding intercept of MBTH versus $1/M_0$ resulted in slope K_m^{MBTH} and the corresponding intercept of P_C versus $1/P_0$ resulted in slope K_m^{PC}.

RESULTS

Experimental Protocol Conducted For the Quantification of Hydrogen Peroxide

The linearity for the assay of H_2O_2 was determined in 3 mL of the solution containing 0.1545 mM MBTH, 0.6054 mM PC, and 1.15 EU of catalase in 50 mM tris buffer at pH 9.8. The reaction was initiated at 30^0 C by adding 100 µL of varying concentrations of H_2O_2 within the linearity range. The change in absorbance was continuously recorded at 500 nm. The initial rate was then plotted against the concentration of H_2O_2 resulted a straight line equation $V_0 = 0.009\ [H_2O_2] + 0.016$ with a correlation coefficient of 0.992. The linearity of the graph was found between 0.6 and 9.62 mM H_2O_2. The calibration graph for the quantification of H_2O_2 is shown in Figure 1.

Experimental Protocol Conducted for the Quantification of Catalase

The change in absorbance with respect to time for the quantification of catalase was measured continuously for 5 min in a 3 mL reaction mixture containing 0.1545 mM MBTH, 0.6054 mM PC, and 9.620 mM H_2O_2 in tris buffer at pH 9.8 were taken. The reaction was initiated by adding 100 µM of varying concentrations of catalase enzyme. The change in absorbance was continuously recorded at 30°C against the corresponding control containing all the reagents except catalase. The initial velocity (V_0 expressed in terms of EU min^{-1}) was recorded by the absorbance-time curve. The rate verses the units of catalase followed a linearity equation $V_0 = 0.067\ CAT_{units} + 0.013$ with a correlation coefficient 0.994. The calibration plot for the quantification of catalase is shown in Figure 2. The range for linear relationship between the initial velocity and the concentration of catalase was 0.14-3.46 EU from the rate method. In fixed time method 10 min of incubation of the reaction mixture containing all the reagents at the same pH at 30°C allowed the catalase to be assayed at concentration range of 0.03-0.35 EU. The linear relationships by the rate and fixed time methods are shown in Figure 3

Unit of Enzyme

One unit of enzyme is defined as that amount of enzyme, which utilizes 1 µmol of H_2O_2 for the coupling of 1 µmol of MBTH and 1 µmol of PC to form red colored coupled product per min under the standard assay conditions.

Protein Determination

The total protein concentration was determined in triplicate by Lowry method (Koc, O. et al, 2010), using bovine serum albumin as standard.

DISCUSSIONS

Absorption Spectrum of Hydrogen Peroxide

The absorption spectrum of the colored solution produced at 2, 4 and 8 mM concentrations of H_2O_2 was measured by the recommended general experimental protocol in a final 3 mL of reaction mixture and the spectrum was recorded at a scan rate of 2 nm/s after incubating the reaction mixture for 5 min at 30 °C on a spectrophotometer in the wavelength range

400–800 nm against the corresponding reagent blank. The optimum wavelength for maximum absorption of the colored product was 500 nm. The spectrum in the absence of H_2O_2 and enzyme showed negligible absorption. The result is graphically presented as the inset in Figure 2.

Optimization of the Experimental Conditions for Maximum Activity of Enzyme Assay

Optimizations of experimental conditions parameters such as effect of substrates, co-substrates, different buffer concentrations of pH 3.5–10.0, temperature and incubation period, which affect enzyme assay, have been studied.

Effect of Hydrogen Peroxide

The effect of different concentrations of H_2O_2 on the rate of reaction was studied, where rate increased linearly up to 9.62 mM concentration of H_2O_2 beyond which the rate increases gradually and become independent of the concentration due to the enzyme getting saturated. Although at higher concentration of H_2O_2, the reaction rate increased, but the change in the rate was very small. Hence it is decided to have a final H_2O_2 concentration of 9.62 mM in 3 mL of the reaction mixture. The effect of H_2O_2 on the rate of reaction is shown as inset of Figure 1.

Effect of MBTH and PC

The effect of varying concentrations of MBTH and PC was studied and the results showed that the rate increased on increasing the concentration of MBTH from 0.0193 mM to 0.1545 mM beyond which it inhibited the rate. Hence for all further assays MBTH concentration of 0.1545 mM was selected. Similarly, the effect of PC concentration on the reaction rate was studied from 0.0378 mM to 2.4216 mM. The linearity was observed up to 0.6054 mM, above this concentration there was no effect on the rate. Hence 0.6054 mM was selected as the optimized concentration for all further analysis. The plots of reaction rate *versus* concentration of MBTH and PC are shown in Figure 4

Effect of Temperature on the Sensitivity of Enzyme Assay

Temperature sensitivity was determined by pre-incubating 0.1545 mM MBTH, 0.6054 mM PC, 9.62 mM H_2O_2, and 1.15 EU catalase in 50 mM tris buffer of pH 9.8 for 10 min at different temperatures (0-80 ^{0}C). The activity of the enzyme was registered as a function of absorbance of the colored solution. The activity initially increased up to 30 ^{0}C and gradually decreased thereafter. Figure 5 depicts the percentage activity at different temperatures with reference to the enzyme activity at 30◦C.

Effect of pH and Concentration of Buffer Solutions

Since the rate of color generation primarily depends on pH, we examined the following buffers at 0.5 M for the assay namely, citric acid/potassium citrate at pH 3.6-5.6, acetate/acetic acid at pH 3.6-5.6, potassium dihydrogen phosphate/sodium hydroxide at pH 6.0-8.0, potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate at pH 6.0-7.5 and a tris buffer at pH 9.8. The highest activity of the enzyme was observed in 50 mM tris buffer of pH 9.8. Hence, further studies were carried out at this pH. The response of the enzymatic activity with reference to pH is shown in Figure 6.

Discussion of Proposed Reaction Pathway for the Enzyme Activity Response

A possible mechanism for the catalase-catalyzed reaction of MBTH and PC in presence of H_2O_2 involved the biocatalatic coupling of the reactants as proposed in Scheme 1. Catalase is relatively unique compared to other members of the peroxidase family since it displays both catalase activity and peroxidase activity (Wu, M. et al, 2003), hence the

enzymatic mechanism is analogous to that suggested by Setti et al (Setti, L. et al, 1998) for the HRP-catalyzed oxidative coupling of MBTH and phenolic compounds with the formation of azo-dye. In the proposed reaction, the process involves the double-displacement mechanism for the H_2O_2 and electron donating phenolic compound (Mello, L.D., et al, 2003). The catalase is first oxidized by H_2O_2 and then reduced by phenolic compounds. The phenolic compounds are converted to quinones (Shivakumara, A., et al, 2011). In the presence of H_2O_2, MBTH undergoes peroxidation reaction to form MBTH radical cation (Nagaraja, P., et al, 2009) which gets coupled with quinone to form a red colored azo-dye product (scheme-1), showing a strong absorption at 500 nm. The kinetic mechanism in which catalytic coupling between MBTH and PC involving different Michaelis-Menten values cannot over rule the activation of both by a bio catalytic mechanism. Both MBTH and PC could be activated on the same or different catalytic sites through reduction, finally involving the reaction between the two activated reactants. The bimolecular reaction catalyzed by an enzyme involves the formation of an intermediate with any one of the reactants, followed by the reaction of the intermediate with another reactant. Either of these two could become a rate-determining factor.

Evaluation of Kinetic Parameters for the Enzyme Assay

Kinetic parameters for the enzyme assay were studied under the optimized experimental conditions. Equation 1 was used to set up the kinetic mechanism and to obtain the Michaelis-Menten constant values for all the substrates. In the proposed assay, evaluation of Michelis-Menten constant of MBTH, various H_2O_2 concentrations at different fixed concentrations of MBTH were used. The MBTH concentrations that were used in the assay are 0.0570, 0.0856, 0.1141 and 0.1426 mM. The rates of the reaction were measured at different H_2O_2 concentrations namely 3.85, 5.77, 7.70 and 9.62 mM with a constant 0.6054 mM PC concentration. The rates of each of the H_2O_2 concentration at one fixed concentration of MBTH were evaluated. The intercept obtained by the plot of the $1/H_0$ versus V_{max}/V_0 of the reaction at different MBTH concentrations were evaluated according to the equation (2), as shown in the figure 7 (panel A). Similarly for the evaluation of Michaelis – Menten constant of PC, various H_2O_2 concentration at different fixed concentration of PC were used. The PC concentrations that were used in the assay 0.1513, 0.3026, 0.4539 and 0.6053 mM. The rates of the reaction were measured at different H_2O_2 concentrations namely 2.40, 4.81, 7.21 and 9.62 mM with a constant 0.1426 mM MBTH concentration. The rates of each of the H_2O_2 at one fixed concentration of PC were evaluated. The intercept obtained by the plot of the $1/H_0$ versus V_{max}/V_0 of the reaction at different PC concentration were evaluated according to the equation (2), as shown in the figure 7 (panel B). The replots of the intercepts of panels A and B of Figure 7 *versus* the reciprocal concentration of H_2O_2 also give a constant slope (figure not shown). The K_m^{MBTH} and K_m^{PC} were found to be 80 µM and 487 µM, respectively. The value of $K_m^{H_2O_2}$ and V_{max} was calculated from the Hanes plot (Figure 8), of H_2O_2 concentration ranging from 0.6 to 38.49 mM. The Hanes plot starts with the Lineweaver – Burk equation,

$$\frac{1}{V_0} = \frac{K_m^{H_2O_2}}{V_{max}} \cdot \frac{1}{S_0} + \frac{1}{V_{max}} \quad \text{Or}$$

$$\frac{[S_0]}{V_0} = \frac{1}{V_{max}}[S_0] + \frac{K_m^{H_2O_2}}{V_{max}}$$

Where slope = $1/V_{max}$ and intercept = $\frac{K_m^{H_2O_2}}{V_{max}}$.

According to Hanes the plot of $[S_0]/V_0$ *versus* $[S_0]$ gives a linear equation $\frac{[S_0]}{V_0} = 7.489[S_0] + 30.71$ with regression co-efficient of 0.993. The $K_m^{H_2O_2}$ and V_{max} values were found to be 4099 µM and 0.1335 EUmin^{-1} respectively. The $K_m^{H_2O_2}$ value of the coupling reaction was 4099 µM, which reflects the higher number of interactions between MBTH - PC and the heme group at the active site. The catalytic efficiency $\frac{1}{slope \times [E]}$, catalytic power $\frac{V_{max}}{K_m}$ and catalytic constant (k_{cat}) $= \frac{V_m}{[E]_t}$ of the proposed MBTH – PC system was calculated by a general kinetic procedure using 0.1545 mM MBTH, 0.6054 mM PC, 50 mM tris buffer of pH 9.8 and 1.15 EU catalase in 3 ml of reaction mixture were found to be 0.11×10^6 M^{-1} min^{-1}, 3.26×10^{-5} min^{-1} and 0.1161 min^{-1}, respectively.

APPLICATION

The developed method was successfully applied to study catalase in some microbes such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Aspergillus flavus.* Microbial inoculums were prepared as described earlier. For the assay of catalase in microbes, we took 0.1545 mM MBTH, 0.6054 mM PC, 9.620 mM H_2O_2 and 100 µL sample after proper dilution with 50 mM tris buffer of pH 9.8 in 3 ml of reaction mixture, as described in the developed procedure, and absorbance was measured at 500 nm. The activity of the sample was quantified by considering the standard absorbance. The accuracy of the proposed method was assessed by comparing the results obtained with a reference CAT assay method (Góth, L., 1991). Also catalytic activity of CAT has been evaluated in the samples. The results obtained by these two methods for the analysis of micro organism samples are indicated in Table 1. Catalytic power, V_{max} and K_m values for the samples are shown in Table 2.

CONCLUSIONS

The developed method is simple, rapid, reliable, and highly sensitive for the assay of catalase in microbes which involves coupling of oxidized PC and MBTH using H_2O_2. Co-substrates used are easily available, economical, water-soluble, have good catalytic efficiency, and the coupled product gets absorbed at a longer wavelength (500 nm) which enables to avoid the background interference caused by the biological constituents than the L. Goth method (Góth, L.,1991). The kinetic study of the system showed instantaneous color formation, and it requires only small quantities of colorimetric reagents. Optimization of the reaction conditions from the enzymatic oxidation allowed the determination of H_2O_2 from 0.6 to 9.62 mM. This catalytic reaction in presence of peroxide allowed spectrophotometric determination of catalase within the linearity range of 0.14-3.45 EU and 0.03 - 0.34 EU by the kinetic and fixed time methods, respectively in 3 ml solution. This linear dependence between the concentration of catalase and the absorbance over a narrow range is also an important feature for the practical application of the assay procedure. The proposed method is more sensitive than the INH- PC system developed earlier by our group [27] in which the linearity of the catalase activity and H_2O_2 were in the range of 0.2–7.0 units and 1.76–7.0 mM, respectively in 3 ml solution. The kinetic study shows that the Michaelis – Menten constant $K_m^{H_2O_2}$ for the projected method is 4.09 mM which is less than our previous

INH – PC system. This indicates that there is stronger affinity of active site of PC and MBTH to that of H_2O_2 molecules which signifies selectivity and specificity of the proposed assay method. This method was successfully applied to the assay of catalase in some selected microorganisms.

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APPENDICES

Table 1: Catalase Activity of Some Micro Organisms as Determined by the Proposed Method and Standard Method Mean (n = 3 Replicate Determinations) ± R.S.D

Sample [a]	Units [b]		Protein [b] (mg)	Specific Activity (Units/ mg of Protein)		Relative Catalytic Efficiency
	Proposed Method	Reference Method		Proposed Method	Reference Method	
Bacillus subtilis	56.50 ± 1.01	55.2 ± 1.21	5.0119 ± 1.08	11.27 ± 1.74	11.01 ± 1.96	4.21
Staphylococcus aureus	3.38 ± 1.22	3.12 ± 1.42	0.3044 ± 1.31	11.10 ± 1.64	10.24 ± 2.21	2.06
Pseudomonas eruginosa	4.48 ± 1.52	4.78 ± 1.86	6.176 ± 1.71	0.72 ± 2.10	0.77 ± 1.54	1.12
Aspergillus flavus	26.25 ± 1.33	25.23 ± 1.62	3.667 ± 1.23	7.16 ± 1.72	6.88 ± 1.87	2.17

[a] The sample was diluted as per the requirement of the assay.
[b] Mean ±RSD, n = 5 determinations.

Table 2: Catalytic Parameters of Some Microorganisms as Determined by the Proposed Method

Organism	V_{max} min^{-1}	$K_m$value µM	Catalytic Power $min^{-1}(10^{-5})$
Bacillus subtilis	0.0466	1556.92	2.9931
Staphylococcus aureus	0.0550	3287.62	1.6729
Pseudomonas aeruginosa	0.1848	10739.74	1.7207
Aspergillus flavus	0.0524	3783.43	1.3850

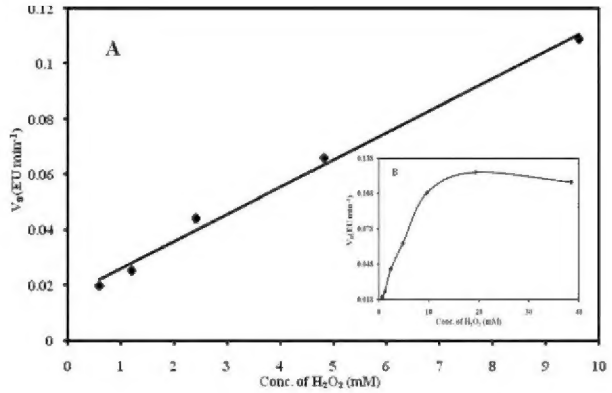


Figure 1: (A). Calibration Graph for the Quantification of H_2O_2 (mM) and Inset (B) Shows the Effect of H_2O_2 Concentration on the Rate of Reaction: 0.1545 mM MBTH + 0.6054 mM PC + 1.15 EU of Catalase + 50 mM Tris Buffer at pH 9.8 with Varying Concentration of H_2O_2

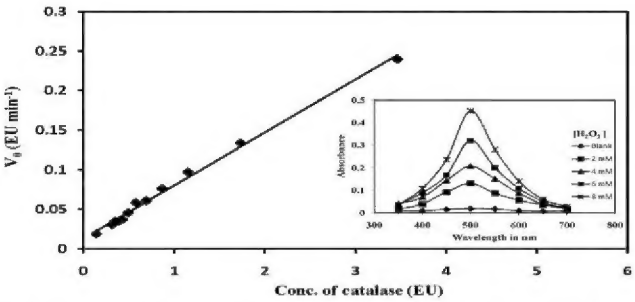


Figure 2: Calibration Graph for the Quantification of Catalase by the Rate Method. The Reaction Mixture Containing 0.1545 mM MBTH + 0.6054 mM PC + 50 mM Tris Buffer at pH 9.8 + 9.6 mM H_2O_2 with Varying Concentration of Catalase. Inset Shows the Absorption Spectrum with Varying Concentration of H_2O_2

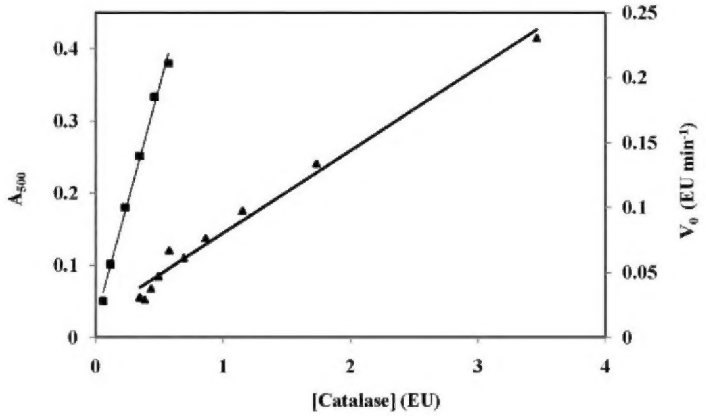


Figure 3: Calibration Graph for the Quantification of Catalase by ▲Rate Method and ■Fixed Time Method

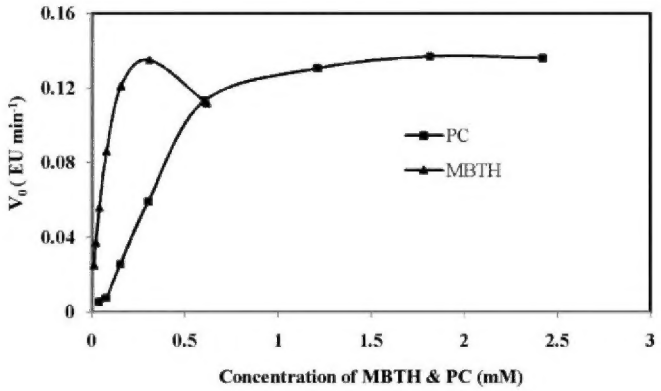


Figure 4: Effect of MBTH and PC on the Reaction Rate

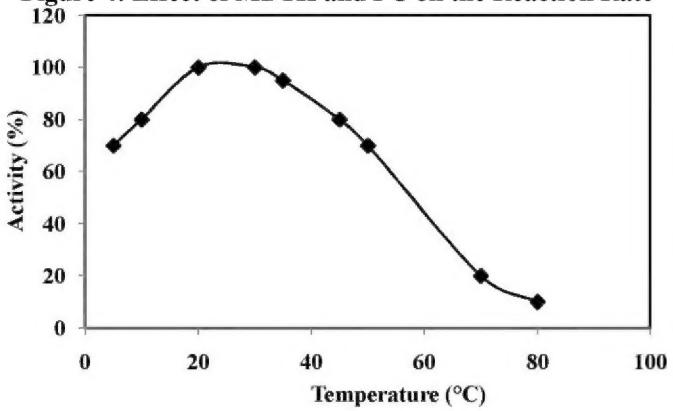


Figure 5: Effect of Incubation Temperature on the Reaction Rate

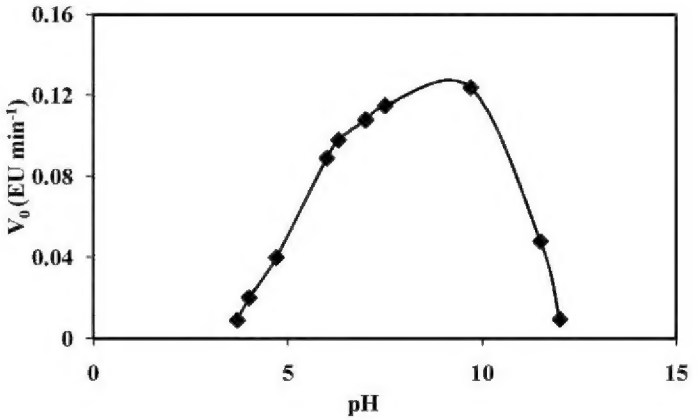


Figure 6: Effect of pH on the Reaction: Citric Acid/Citrate Buffer (pH 3.6–5.3), and Acetate/Acetic Acid Buffer (pH 3.6–5.6), Potassium Dihydrogen Orthophosphate/ Sodium Hydroxide Buffer (pH 5.8–7.8), and Potassium Dihydrogen Orthophosphate/ Dipotassium Hydrogen Phosphate Buffer (pH 6.0–7.5) (N)

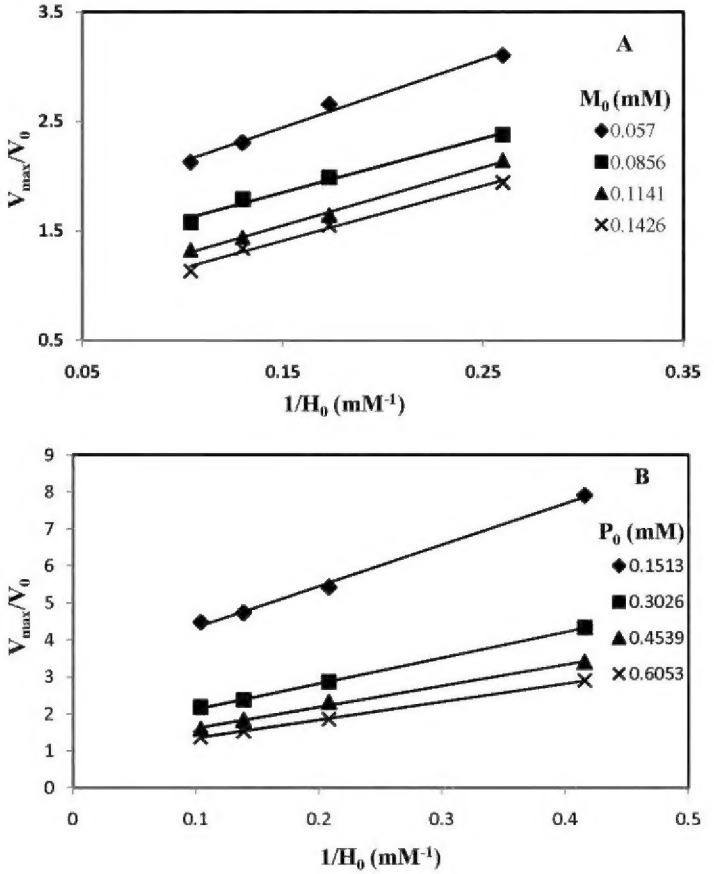


Figure 7: Kinetic Behavior of Two Substrate Reactions for Pure Catalase (1.15 EU). Plot of the Substrate-Velocity Relationship, According to eq. 3 for (A) MBTH and (B) PC, with Different H_2O_2 Concentration

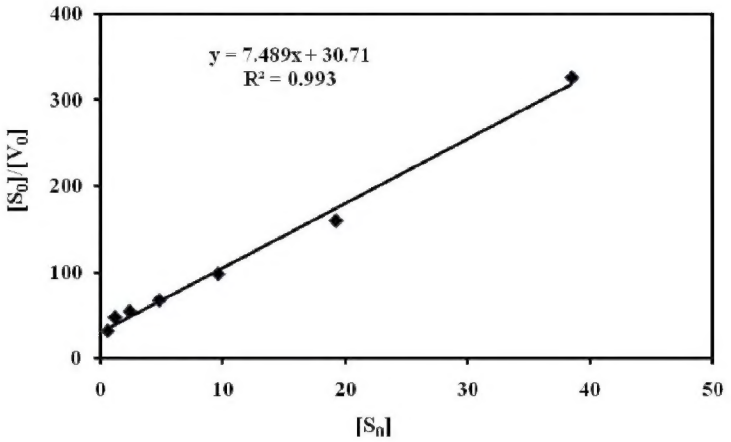
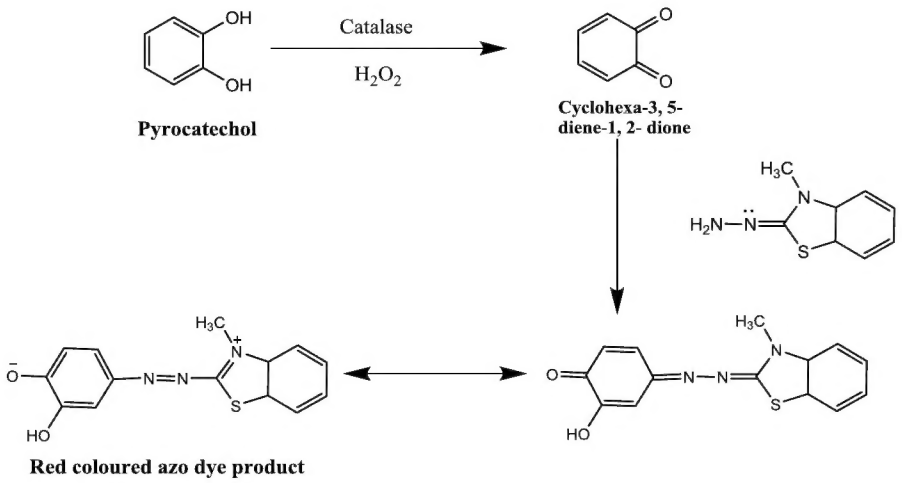


Figure 8: Hanes Plot for Catalase by the Proposed Method. The Kinetic Study was Carried Out for 1.15 EU Catalase as Discussed in Materials and Methods



Scheme 1: Probable Reaction Pathway for the Formation of a Red Colored Product by Coupling of MBTH and PC in Presence of H_2O_2 and Catalase